Di-2-ethylhexyl Phthalate in Bovine Heart Muscle Mitochondria: Its Detection, Characterization, and Specific Localization

by Darius Jal Nazir,* Morton Beroza,† and Padmanabhan P. Nair‡

During our studies on the fatty acid composition of several lipid classes of subcellular fractions of bovine heart muscle, a peak which constituted 60% of the total fatty acids of mitochondrial triglycerides was consistently observed in GLC runs *1,2)*. The earlier assumption that this compound was either an artifact or a contaminant was inconsistent with the manner in which this compound consistently appeared in such proportions only in this single subcellular fraction. The experiments to isolate this component of mitochondrial triglycerides and identify it as di-2-ethylhexyl phthalate (DEHP) are described in this presentation.

Subcellular Fractionation

Fresh beef heart muscle was trimmed of all excess fat and homogenized in an EDTA–sucrose solution. Figure 1 shows the procedure used for isolating the nuclear, heavy and light mitochondrial, microsomal, and cytoplasmic fractions by differential centrifugation *(3–5)*. All the subcellular fractions (after purification) were essentially homogenous by electron microscopy.

Extraction and Column Chromatography

The lipids of all the subcellular fractions were extracted according to the procedure described by Folch *(5, 6)* and chromatographed on silicic acid *(7–9)* (Table 1). The unknown compound was eluted in the 4% ether in hexane fraction, together with the triglycerides.

Transmethylation

The fatty acids in the lipid fractions were transmethylated in the presence of methanol, hydrochloric acid, and dimethoxypropane *(8)*, and the resulting methyl esters were analyzed by gas-chromatographic techniques.

Gas-Liquid Chromatography

The methylated samples were run on three phases: a polar 16% diethyleneglycol succinate (DEGS) column, a relatively nonpolar 8% Apiezon L column, and a two-phase SE52-XE60 column. Only the triglyceride fraction of the lipid extracted from heavy mitochondria showed the pres-

*McMaster Medical Unit, Hamilton General Hospital, Hamilton 21, Ontario, Canada.
†Entomology Research Division, U.S. Department of Agriculture, Beltsville, Maryland 20705.
‡Biochemistry Research Division, Sinai Hospital of Baltimore, Inc., Baltimore, Maryland 21215.

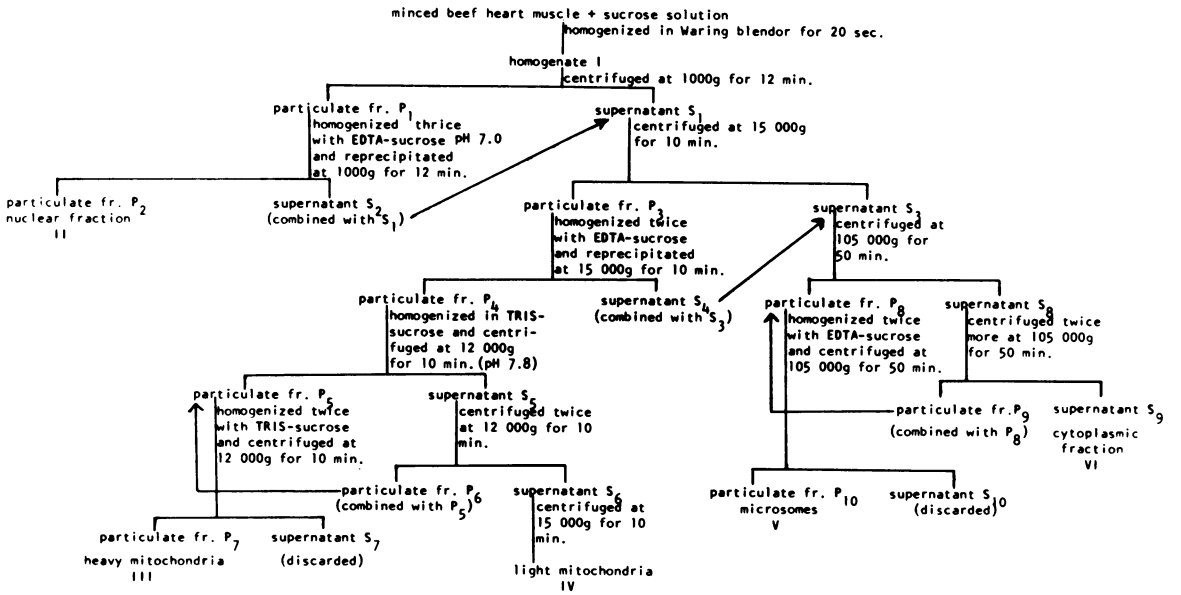


FIGURE 1. Separation of beef heart muscle homogenate into subcellular fractions (the centrifugal forces are given as R_{max}).

ence of this unknown peak in appreciable amounts. It had a retention time 13.6, 4.4, and 4.6 times that of methyl octadecanoate on these three phases, respectively (Table 2).

Preparative-Scale Gas-Liquid Chromatography

The unknown compound was isolated from the mitochondrial triglyceride fraction by using a 10 ft x 8 mm DEGS column maintained at 200°C and at an argon gas pressure of 30 psi.

Elemental Analysis

A quantitative determination of the elements present in this compound showed 73.64% of carbon, 9.64% of hydrogen and 16.70% of oxygen, which agreed closely with the calculated values for $C_{24}H_{38}O_4$ (Table 3).

Microhydrogenation

In order to get some idea of the number of double bonds present in the unknown compound about 2 μg was subjected to microhydrogenation as described by Beroza and Sarmiento *(10)* (Table 4). A tubular by-pass splitter was used; the catalyst was "neutral" 1% palladium on 99% Gas Chrom P. Hydrogen was the obligatory carrier gas, while nitrogen was introduced into the port at the flame head where hydrogen usually enters in ordinary gas chromatography. The vapor emerging from the hydrogenator was passed through a DEGS column. Since no change was observed on passing the sample through the hydrogenator at 200°C, it was probable that no aliphatic double bonds were present.

Microozonization

Another 5-μg aliquot of the unknown compound was subjected to microozonization as described by Beroza and Bierl *(11)*. The compound was held at −60°C in a solvent like methylene chloride during ozonization. Excess ozone resulted in the appearance of side products, and therefore the reaction was stopped as soon as the gas emerging from the reaction chamber contained ozone. The ozonized sample was subjected to gas chromatography on a DEGS column. No fragments were observed during the gas chromatographic run, and once again only a single peak was obtained, correspond-

Table 1. Steps in isolation of unknown compound from beef heart muscle mitochondria.

Step			
1.	Subcellular fractionation		
2.	Extraction of fractions with chloroform: methanol (2:1)		
3.	Chromatography of silicic acid		
	Solvent system	Lipid class	Volume of eluent, ml
	Hexane	Free aldehydes hydrocarbons and degradation products	200
	1% Ether in hexane	Cholesterol esters	500
	4% Ether in hexane	Triglycerides + unknown compound	600
	8% Ether in hexane	Free fatty acids	250
4.	Methylation of lipid from 4% ether in hexane fraction		
5.	Gas-liquid chromatography of methylated fatty acids		
6.	Isolation by preparative gas-liquid chromatography on a 16% diethylene glycol succinate column		

Table 2. Retention time data on the unidentified component from bovine heart muscle mitochondrial triglyceride relative to methyl octadecanoate.[a]

Stationary phases	Unknown compound	DEHP
Diethyleneglycol succinate[b]	13.6	13.5
Apiezon L[c]	4.4	4.4
SE52/XE60 (2:1)[d]	4.6	4.6

[a] Methyl octadecanoate = 1.00

[b] Column temperature: 180°C; outlet flow rate; 75 ml/min.

[c] Column temperature: 200°C; outlet flow rate; 300 ml/min.

[d] Column temperature: 200°C; outlet flow rate; 250 ml/min.

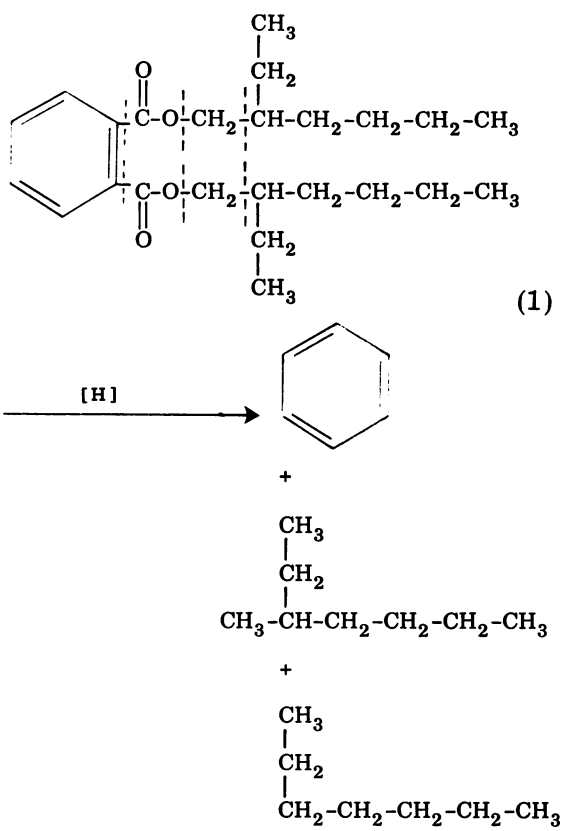
Table 3. Elemental Analysis: $C_{24}H_{38}O_4$.

	C, %	H, %	O, %
Calculated	73.85	9.74	16.41
Found	73.64	9.64	16.70

ing to the original sample in both retention time and peak area. The result again indicated the absence of aliphatic double bonds.

Carbon Skeleton Chromatography

The unknown compound was subjected to carbon skeleton chromatography *(12, 13)*. The tube containing the catalyst (1% palladium on Gas Chrom P) was screwed onto the injection port of a gas chromatograph. The carrier gas hydrogen swept the sample over the catalyst whose temperature was maintained at 300° C. This resulted in the saturation of multiple double bonds, if present, and the replacement of halogen, oxygen, nitrogen, and sulfur atoms by hydrogen. The unknown compound gave peaks with retention times corresponding to *n*-heptane (11.9 min), a branched-chain hydrocarbon having eight carbon atoms (3-methylheptane, which is 2-ethylhexane) (20.0 min), and a weak peak for benzene (9.5 min). The breakdown pattern and the relative proportions in which each of the hydrocarbons was obtained suggested the presence of a disubstituted benzene ring with a 2-ethylhexyl side chain in the original molecule [eq. (1)]. Authentic



(1)

Table 4. Analytical procedures for the identification of di-2-ethylhexyl phthalate.

Step	Procedure	Result	Reference
1.	Hydrogenation followed by GLC	No change in retention time	(*10*)
2.	Microozonization followed by GLC	No fragments produced	(*11*)
3.	Carbon-skeleton chromatography	Revealed heptane, 2-ethylhexane, and benzene (weak)	(*12, 13*)
4.	Saponification (strong alkali)	A. Nonsaponificable fraction yielded 2-ethyl-1-hexanol, identified by GLC	
		B. Saponifiable fraction, after methylation, gave dimethyl phthalate, identified by GLC	

DEHP gave products with retention times identical to those obtained with the unknown compound. These results indicated that the unknown compound was probably di-2-ethylhexyl phthalate. To show that it was, the isolated compound and standard DEHP were subjected to gas chromatography, separately and together. The retention times were identical. The identity of this compound was further confirmed by other experimental evidence.

Saponification

The nonsaponifiable portion obtained after drastic alkaline hydrolysis with 30% aqueous KOH for 2 hr contained a single component, having a retention time identical to 2-ethyl-1-hexanol on DEGS, Apiezon L, and SE52-XE60 columns (Table 5).

The acidic portion, when subjected to gas chromatography after methylation, gave only one peak with the same retention characteristics as *o*-dimethyl phthalate on all three phases (Table 6). These results again indicated that the unknown compound was di-2-ethylhexyl phthalate.

Infrared Spectrum

The infrared spectrum of the isolated component showed a band at 13.5 μ, indicating the presence of an *ortho*-disubstituted benzene ring (Fig. 2). Absorption bands at 7.9, 8.9, and 9.4 μ were attributed to carbon–oxygen absorption and are one of the characteristics of phthalates; the band at 5.8 μ was attributed to the ester carbonyl; at 6.9 μ to the aliphatic C-H; and at 7.3 μ to the methyl groups. The ratios indicated

Table 5. Retention time data for several closely related alcohols on three different stationary phases.

Stationary phase	Retention time, min							
	1-Butanol	Isobutyl alcohol	Isoamyl alcohol	4-Heptanol	2-Ethyl-1-hexanol	1-Octanol	2-Octanol	Unknown[a]
Diethylene glycol succinate	3.1	2.2	4.3	6.8	20.5	30.7	14.7	20.5
Apiezon L			1.7	9.3	25.2	35.3	21.4	25.2
SE52-XE60			2.0	6.4	17.4	24.9	13.6	17.4

[a]Unknown sample represents the alcohol moiety of the compound isolated from bovine heart muscle mitochondria.

Table 6. Retention time data of *o*-, *m*-, and *p*- dimethyl esters of benzenedicarboxylic acids on three different stationary phases.

Stationary phase	Retention time, min			
	Ortho	*Meta*	*Para*	Unknown[a]
Diethylene glycol succinate	14.2	12.9	11.7	14.2
Apiezon L	2.4	3.8	3.8	2.4
SE52-XE60	2.3	2.7	2.5	2.3

[a] Unknown sample represents the methylated acid moiety of the compound isolated from bovine heart muscle mitochondria.

that the alcohol moiety was longer than propyl, and possibly branched. These data were consistent with the pattern expected from a compound with the structure of DEHP.

Mass Spectrum

The mass spectrum of the isolated compound was identical with that of standard DEHP (Fig. 3). Some of the peaks in this figure have been amplified off scale in order to show the parent peaks better. The molecular formula established by peak matching the molecular ion $C_{24}H_{38}O_4+$ was found to be 390.2765, compared to a theoretical value of 390.2770. The *m/e* values of fragments with the highest relative abundance corresponded to rearrangement ions of phthalic anhydride (149; M-241); the diacid fragment (167; M-223); the one derived by the loss of a 2-ethylhexyl group from the parent molecular ion (279; M-111); and a 2-ethylhexane hydrocarbon fragment (112; M-278).

Nuclear Magnetic Resonance Spectrum

The NMR spectrum showed a pair of overlapping triplets centered about 9.1, overlapping multiplets with the strongest peak at 8.7, a doublet at 5.9, and another multiplet centered at 2.5 (Fig. 4). The chemical shifts have been expressed in parts per million relative to tetramethylsilane at 10 ppm (τ scale). These data were consistent with the struc-

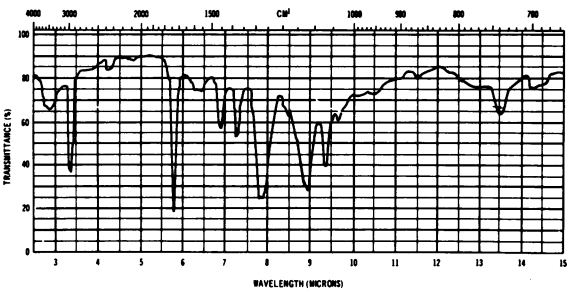


FIGURE 2. Infrared spectrum of di-2-ethylhexyl phthalate.

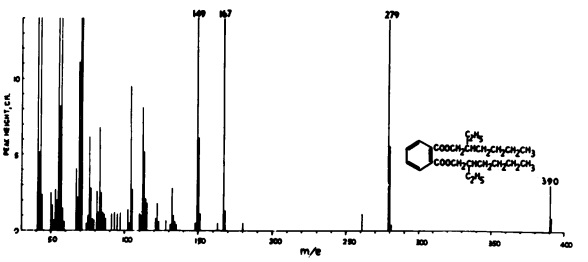


FIGURE 3. Mass spectrum of di-2-ethylhexyl phthalate.

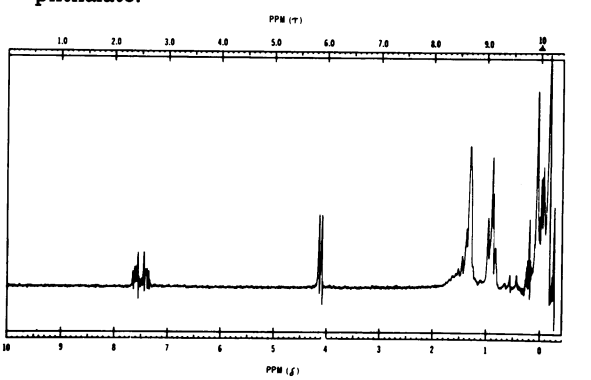


FIGURE 4. Nuclear magnetic resonance spectrum of di-2-ethylhexyl phthalate.

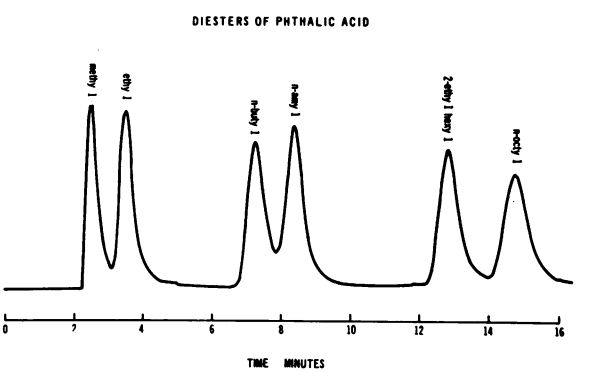


FIGURE 5. Gas-liquid chromatographic separation of the diesters of phthalic acid.

tural features of DEHP and were identical to that obtained with the standard compound.

Gas-Liquid Chromatography of Diesters of Phthalic Acid

The separation of several diesters of phthalic acid on a 5% SE30 column is shown in Figure 5. Although Perkins *(14)* was unable to distinguish between di-2-ethylhexyl phthalate and di-*n*-octyl phthalate, our results show that these compounds could be easily separated on a non-polar column.

Subcellular Distribution in Heart Muscle Homogenate

The triglyceride fraction of the total lipids obtained from the nuclear, heavy and light mitochondrial, microsomal and cytoplasmic fractions of bovine heart muscle homogenate was transmethylated and subjected to gas chromatography on a mixed-phase SE52-XE60 column to quantitate the DEHP (Table 7). The quantitation of DEHP in the nuclear fraction was rendered inaccurate due to the extremely low levels of DEHP in relation to the other lipids in this fraction. The DEHP obtained from the heavy mitochondrial fraction accounted for more than 99.5% of the total DEHP present in the cell.

Table 7. Distribution of di-2-ethylhexyl phthalate (DEHP) among subcellular fractions from bovine heart muscle.

Subcellular fraction	DEHP, mg/100 g muscle	DEHP, mg/100 g total lipid [a]	DEHP, mg/100 g total triglyceride [b]
Nuclear	Trace [c]	—	—
Heavy mitochondrial	13.5	304.1	1752.6
Light mitochondrial	0.013	0.293	1.689
Microsomal	0.015	0.338	1.948
Cytoplasmic	0.033	0.743	4.283

[a] Represents the DEHP in 100 g of total lipid from whole heart homogenate, distributed among subcellular fractions. Values were computed from total lipid content of whole heart muscle (4.44 g/100 g muscle).

[b] Represents the DEHP in 100 g of total triglyceride from whole heart homogenate, distributed among subcellular fractions. Values were computed from total triglyceride content of whole heart muscle (0.77 g/100 g muscle).

[c] DEHP was found only in traces and could not be quantitatively determined.

DEHP in Heart Muscle of Other Species

Subcellular fractionation of the heart muscle of the rat, rabbit, and dog was carried out as described for bovine heart muscle. Once again DEHP was present only in heavy mitochondrial triglycerides, but in amounts much smaller than that found in beef heart mitochondria (Table 8). No DEHP was detected in the triglycerides of the other subcellular fractions of heart muscle of these species.

Johnson and Roots *(15)* have suggested that anhydrous methanolic HCl may be a source of such artifacts as the esters of carboxylic acids formed during methanolysis. These artifacts were assumed to have been formed either by the oxidative breakdown of methanol coupled with condensation reactions, or simply to have been present as contaminants in the methanol. However, we were able to recover DEHP quantitatively by direct gas–liquid chromatography of mitochondrial triglycerides without prior transmethylation, and consequently any possibility of DEHP arising as an artifact during methanolysis was eliminated. Under our conditions of esterification, DEHP was not attacked. Moreover, if DEHP was an artifact formed during the experimental procedure, it should have appeared in all fractions; the repeated observations that DEHP was present in only one out of the fourteen lipid fractions isolated by silicic acid chromatography would rule out this possibility.

DEHP is a compound widely used as a plasticizer in the production of tubing,

Table 8. Di-2-ethylhexyl phthalate in heart muscle mitochondria from other species.

Species	DEHP, μg[a]
Rat	129
Rabbit	118
Dog	36

[a] Expressed as μg of DEHP in mitochondria derived from 100 g of original heart muscle.

synthetic resins, flexible films, and as an additive in vacuum pump oils. Recent reports have indicated that DEHP is present, sometimes in relatively high concentrations, in milk *(16)* and blood *(17)* stored in containers made of synthetic rubber and plastic, presumably due to a leaching effect exerted by the liquid. Yoshinori and Fumihide *(18)* have reported the presence of DEHP in some commercial solvents. However, our complete control runs using the same redistilled solvents and other reagents were essentially negative, showing that DEHP did not originate as a contaminant from laboratory reagents or equipment. All possible sources of contamination were scrupulously avoided during the processing of our samples. In order to avoid any possible contamination from polyethylene ultracentrifuge cups, subcellular fractionation up to the stage of the postmitochondrial supernatant was performed in stainless steel containers in certain experiments.

At this time there is no firm evidence to show whether heart mitochondria could possibly biosynthesize molecules like DEHP from simpler compounds or whether they arise from dietary sources and become specifically localized in this subcellular organelle. Esters of phthalic acid have been reported to have been formed by thermal oxidation, *in vitro*, of corn oil and triolein, and Perkins *(14)* has speculated on the chemical reactions leading to the aromatization of linoleic and arachidonic acids.

Evidence from other laboratories shows that compounds like terephthalic acid, if present in the animal body, are rapidly eliminated *(19, 20)*, but more recent reports indicate that DEHP is not attacked by nonspecific esterases *(21)*, and may thus conceivably accumulate in animal tissues.

Our original observation of the presence of DEHP in bovine heart muscle mitochondria has been extended more recently to heart mitochondria of other species such as the rat, rabbit, and dog. However, from our preliminary work on other organs, we have not been able to demonstrate the presence of DEHP in appreciable concentrations, in tissues which are not related to the cardiovascular system.

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